GALACTANASE VARIANTS

FIELD OF THE INVENTION

The present invention relates to variants of galactanases of Glycoside Hydrolase Family 53, their production, and their use within the dairy industry.

BACKGROUND OF THE INVENTION

Background art

The crystallization and preliminary X-ray studies of the galactanase from *Aspergillus aculeatus* is described by Ryttersgaard et al in Acta. Cryst. (1999), D55, 929-930.

SUMMARY OF THE INVENTION

The invention provides variants of a parent Glycoside Hydrolase Family 53 galactanase, comprising an alteration in at least one of the following positions: -6, -4, -2, 1, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 18, 20, 22, 24, 25, 26, 29, 30, 31, 32, 36, 39, 40, 41, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 54a, 54e, 54f, 54g, 54h, 55, 56, 57, 58, 61, 62, 65, 69, 77, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 101, 106, 107, 110, 113, 114, 126, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 144, 145, 146, 147, 150, 153, 157, 159, 163, 169, 171, 172, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 191, 192, 194, 198, 200, 203, 204, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 252, 252d, 252e, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 264, 265, 266, 273, 274, 276, 277, 280, 283, 284, 286, 288, 288a, 289, 292, 293, 294, 295, 296, 297, 298, 299, 300, 301, 302, 302a, 302d, 302j, 302k, 302m, 302n, 302o, 302q, 302r, 302s, 302t, 302u, 302v, 302x, 302y, 302z, 302aa, 302bb, 302cc, 302dd, 302ee, 302ff, 302gg, 302hh, 302ii, 302jj, 303, 304, 305, 306, 307, 308, 309, 310, 311, 312, 313, 314, 315, 316, 317, 318, 319, 320, 321, 322, 323, 324, 325, 326, 327, 328, 329, and 330; wherein

(a) the alteration(s) are independently (i) an insertion of an amino acid immediately downstream of the position, (ii) a deletion of the amino acid which occupies the position, and/or (iii) a substitution of the amino acid which occupies the position; and (b) the variant has galactanase activity.

BRIEF DESCRIPTION OF DRAWINGS

Fig. 1 shows the coordinates for the 3D structure of a GH Family 53 galactanase from *Myceliophthora thermophila* having SEQ ID NO: 1;

Fig. 2 shows the coordinates for the 3D structure of a GH Family 53 galactanase from *Humicola insolens* having SEQ ID NO: 2;

Fig. 3 shows the coordinates for the 3D structure of a GH Family 53 galactanase from *Aspergillus aculeatus* having SEQ ID NO: 3;

Fig. 4 shows the coordinates for the 3D structure of a GH Family 53 galactanase from *Bacillus licheniformis* having SEQ ID NO: 4;

Fig. 5 shows a multiple alignment of SEQ ID NOs: 1-4; and

Fig. 6 shows the alignment of Fig. 5 with three additional galactanase sequences added.

DETAILED DESCRIPTION OF THE INVENTION

3D-structure determination

The crystallization and preliminary X-ray studies of the galactanase from *Aspergillus aculeatus* (AAGAL) is described by Ryttersgaard et al in Acta. Cryst. (1999), D55, 929-930. The galactanases from *Myceliophthora thermophila* (MTGAL) and *Humicola insolens* (HIGAL) (WO 97/32014), and the galactanase from *Bacillus licheniformis* (BLGAL) (WO 00/47711) were crystallized using similar principles.

The 3D-structures were solved in accordance with the principles for X-ray crystallographic methods as given, for example, in X-Ray Structure Determination, Stout, G.K. and Jensen, L.H., John Wiley & Sons, Inc. NY, 1989. The structural coordinates for the crystal structure of the *Aspergillus aculeatus* galactanase (AAGAL), as determined by multiple isomorphous replacement to 1.8 Å resolution at 100 K are given in Fig. 1 in standard PDB format (Protein Data Bank, Brookhaven National Laboratory, Brookhaven, CT).

The structures of the other three galactanases were solved by Molecular Replacement, using the AAGAL293 structure (to 2.3 Å resolution at 293K) as a search model. Data from 20-2.55 Å, 18-2.14 Å, and 19.67-2.60 Å were used for HIGAL, MTGAL and BLGAL, respectively, within AMoRe (J. Navaza: AMoRe: an Automated package for Molecular Replacement. Acta Crystallogr., A50:157-163, 1994). The respective coordinates are given in Figs. 2-4 in standard PDB format.

Variant

The term "galactanase variant," or simply "variant," refers to a galactanase comprising one or more alteration(s), such as substitution(s), insertion(s), deletion(s), and/or truncation(s) of one or more specific amino acid residue(s) in one or more specific position(s) in a parent galactanase.

The total number of such alterations is typically not more than thirty, e.g. one, two, three, four, five, six, seven, eight, nine, ten, eleven, twelve, thirteen, fourteen, fifteen, sixteen, seventeen, eighteen, nineteen, twenty, twenty-one, twenty-two, twenty-three, twenty-four, twenty-five, twenty-six, twenty-seven, twenty-eight, twenty-nine, or thirty of said alterations.

In addition, the variant of the invention may include other modifications of the parent enzyme, typically not more than 10, e.g. not more than 5 such modifications.

Nomenclature and conventions for designation of variants

A substitution in a variant is indicated as "original amino acid - position - substituted amino acid." The one letter code is preferably used, but it can of course be translated into the three letter code as desired. The codes X (or Xaa) may be used to indicate any amino acid residue. Accordingly, the notation "D182N" or means, that the variant comprises a substitution of aspartic acid with asparagine acid in the variant amino acid position corresponding to the amino acid in position 182 in MTGAL, when the two are aligned as indicated in Fig. 5.

Where the original amino acid residue may be any amino acid residue, a short hand notation may at times be used indicating only the position, and the substituted amino acid, for example: "Position - substituted amino acid", or "182N". This notation is particular relevant in connection with modification(s) in a series of homologous polypeptides, such as the galactanases of GH Family 53. Similarly when the identity of the substituting amino acid residue(s) is immaterial: "Original amino acid - position;" or "D182".

When both the original amino acid(s) and substituted amino acid(s) may be any amino acid, then only the position is indicated, e.g. "182".

When the original amino acid(s) and/or substituted amino acid(s) may comprise more than one, but not all amino acid(s), then the amino acids are listed, separated by commas: "Original amino acid - position no. - substituted amino acid"; e.g. "H91D,L,N".

A number of examples of this nomenclature are listed below:

The substitution of aspartic acid for asparagine in position 182 is designated as D182N.

The substitution of any amino acid residue for serine in position 131 is designated as S131X, or S131.

The substitution of proline for any amino acid residue in position 29 would thus be designated X29P, or 29P.

For a modification where the original amino acid(s) and/or substituted amino acid(s) may comprise more than one, but not all amino acid(s), the substitution of aspartic acid, leucine, or asparagine for histidine in position 91 would be indicated by H91D,L,N; which indicates the specific variants H91D, H91L, or H91N.

A deletion of glutamic acid in position 288a will be indicated by E288a*. Correspondingly, the deletion of more than one amino acid residue, such as the deletion of glutamic acid and aspartic acid in positions 252a and 252b will be designated"E252a*+D252b*"

A truncation means an N- or C-terminal shortening of the complete amino acid sequence, i.e. a deletion of one, or usually more, amino acids and the N- or C-terminal end of the peptide. As regards the designation of truncated variants, the general rule for deletions may be used.

The insertion of an additional amino acid residue such as e.g. a valine after F216 is indicated by "F216FV"; or, when more than one amino acid residue is inserted, such as e.g. a valine, alanine, serine, threonine and a glycine after F216 this will be indicated as:"F216FVASTG".

In such cases the inserted amino acid residue(s) are numbered by the addition of lower case letters to the position number of the amino acid residue preceding the inserted amino acid residue(s). In the above example the sequences would thus be:

Parent:	Variant:						
216	216	216a	216b	216c	216d	216e	217
F	F	V	A	S	T	G	Y

Once all lower case letters from a to z (a,b,c,d,e,f,g,h,i,j,k,l,m,n,o,p,q,r,s,t,u,v,x,y,z) have been used for this purpose, double letters aa, bb, cc etc. onto zz are used, see e.g. the alignment of Fig. 5, between positions 302 and 303.

In cases where an amino acid residue identical to the existing amino acid residue is inserted, it is clear that degeneracy in the nomenclature arises. If for example a phenylalanine would be inserted after the phenylalanine in the above example this would be indicated by "F216FF".

Given that a proline is present in position 215, the same actual change could just as well be indicated as "P215PF":

	Parent:		Variant:		
Numbering I:	215	216	215	216	216a
Sequence:	P	F	P	F	F
Numbering II:			215	215a	216

Such instances will be apparent to the skilled person, and the indication "F216FF" and corresponding indications for this type of insertions is thus meant to comprise such equivalent degenerate indications.

By analogy, if amino acid sequence segments are repeated in the parent galactanase and/or in the variant, it will be apparent to the skilled person that equivalent degenerate indications are comprised, also when other alterations than insertions are listed such as deletions and/or substitutions. For instance, the deletion of two consecutive amino acids "DG" in the sequence "DGDG" from position 252b-252e, may be written as "D252b*+G252c*" or "D252d*+G252e*" or "G252c*+D252d*":

	Parent:				Variant:	
Numbering I:	252b	252c	252d	252e	252b	252c

Sequence:	D	G	D	G	D	G
Numbering II:					252d	252e
Numbering III:					252b	252e

Variants comprising multiple modifications are separated by pluses, e.g. "A90S+H91D"

representing modifications in positions 90 and 91 substituting tyrosine and glutamic acid for arginine and glycine, respectively. Thus, "A90S+H91D,N,L" designates the following variants: A90S+H91D, A90S+H91N, and A90S+H91L. Likewise, N303D,H+N305D,H,P designates the following variants: N303D+N305D; N303D+N305H; N303D+N305P; N303H+N305D; N303H+N305H, and N303H+N305P.

This nomenclature is particular relevant relating to modifications aimed at substituting, inserting or deleting amino acid residues having specific common properties, such modifications are referred to as conservative amino acid modification(s). Examples of conservative modifications are within the group of basic amino acids (arginine, lysine and histidine), acidic amino acids (glutamic acid and aspartic acid), polar amino acids (glutamine and asparagine), hydrophobic amino acids (leucine, isoleucine and valine), aromatic amino acids (phenylalanine, tryptophan and tyrosine), and small amino acids (glycine, alanine, serine, threonine and methionine). Amino acid modifications, which do not generally alter the specific activity are known in the art and are described, for example, by H. Neurath and R.L. Hill, 1979, In, The Proteins, Academic Press, New York. The most commonly occurring exchanges are Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Ser/Gly, Tyr/Phe, Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile, Leu/Val, Ala/Glu, and Asp/Gly as well as the in reverse.

For the present purposes, the sequence of MTGAL (SEQ ID NO:1) has been selected as the frame of reference, meaning that all variants will be defined on the basis of the amino acid sequence of MTGAL. In particular, each amino acid residue in a galactanase sequence is assigned a number, a position, or a position number, by reference to Fig. 5 herein, viz. the number of the corresponding amino acid residue in the *Myceliophthora thermophila* galactanase backbone (MT; the uppermost line of the alignment of Fig. 5). In this context, the term "corresponding" refers to the amino acid which, according to the alignment, is in the same column as the amino acid residue in question, but in the first row designated "MT".

For example, the variant of the galactanase from *Bacillus licheniformis* (BL) which by reference to SEQ ID NO: 4 may be designated S39C will, for the present purposes, be designated S18C, because S39 of BL corresponds to A18 of MT. As another example, the variant of the galactanase from *Aspergillus aculeatus* which by reference to SEQ ID NO: 3 may be designated D182N will, for the present purposes, be designated D181N, because D182 of AA corresponds to N181 of MT. As a still further example, variant K16P of BL may

be designated *-6P, because K16P of BL corresponds to a missing or deleted amino acid in position -6 of MT, still by strict formal reference to Fig. 5.

However, if desired, the variants of the invention may also be defined by reference to their respective "own" backbone, e.g. with reference to SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO: 3, or SEQ ID NO: 4. The corresponding position numbers are easily deduced, in the same way as described above, from Figs. 5-6 or, for additional galactanase sequences, from a figure which can be prepared according to the principles described herein.

Molecular Dynamics (MD)

Molecular Dynamics (MD) simulations are indicative of the mobility of the amino acids in a protein structure (see McCammon, JA and Harvey, SC., (1987), "Dynamics of proteins and nucleic acids", Cambridge University Press). Such protein dynamics are often compared to the crystallographic B-factors (see Stout, GH and Jensen, LH, (1989), "X-ray structure determination", Wiley). By running the MD simulation at, e.g., different temperatures, the temperature related mobility of residues is simulated. Regions having the highest mobility or flexibility (here isotropic fluctuations) may be suggested for random mutagenesis. It is here understood that the high mobility found in certain areas of the protein, may be thermally improved by substituting these residues.

Variants of amended properties

Based on the 3D-structure of the galactanase from *Myceliophthora thermophila* of SEQ ID NO:1, the following variants are contemplated, in which at least one of the below-mentioned residues have been amended and/or at least one of the below-mentioned alterations have been introduced:

i) variants of an amended specific activity, within 10Å from the active site: Y4, G6, V7, D8, W9, S10, R45, Q46, R47, W49, Y77, D79, F80, H81, Y82, W86, A87, D88, P89, A90, H91, Q92, T93, S131, I132, G133, N134, E135, I136, R137, A138, G139, L140, L141, W142, G145, R146, T147, I153, L157, M176, I177, H178, L179, D180, N181, G182, W183, T187, Q188, W191, Y192, M209, G210, V211, S212, F213, Y214, P215, F216, Y217, A221, L226, I241, A242, V243, V244, E245, T246, N247, W248, F276, I277, V280, V284, G292, L293, F294, Y295, W296, E297, P298, W300, L306, G307, F329;

ii) variants of an amended activity on lactose, within 10Å from the active site: Y214S,N+N247Y+L306Q; Y214A; F216FVASTGY217; P89W+W86N;

iii) variants of an amended pH-activity profile: H91N,L,D; N313D; N303D,H; N305D,H; A90S+H91D;

iv) variants of an amended thermostability, by insertion of prolines: Y22P, N24P, T25P, A29P, A53P, N56P, T93P, D101P, W142P, T147P, Q198P, L203P, S204P, S219P, S258P, S288P, A304P, A311P, Q318P, A322P, S324P, S325P, S327P;

v) variants of an amended thermostability, by increasing surface hydrophobicity: W107S,H;

vi) variants of an amended thermostability, by amending the surface electrostatic potential: Q126E;

vii) variants of an amended thermostability, by disulfide bridges (double mutations to cysteines): V20C+G320C, N39C+L326C, Y110C+G163C, W150C+N194C, T274C+V328C, I301C+F316C

viii) variants of amended thermostability, by improved side-chain packing: 9F,Y,W; 12V, 80F, 82Y, 191Y,W; 213F; 9W+12V; 80F+82Y.

Based on the 3D-structure of the galactanase from *Humicola insolens*, the following variants are contemplated, in which at least one of the below-mentioned residues have been amended and/or at least one of the below-mentioned alterations have been introduced:

i) variants of an amended thermostability, by insertion of prolines: V20P, V25P, E29P, V41P, V50P, W53P, N56P, T94P, A96P, W142P, L169P, W185P, Q198P, M203P, A219P, A221P, T222P, Q258P, A261P, D262P, S288P, N305P, A311P, A322P, S324P, S325P.

ii) variants of an amended thermostability, by disulfide bridges (double mutations to cysteines): T113C+G163C, W185C+S229C, S218C+A221C, R227C+V283C.

Based on the 3D-structure of the galactanase from *Aspergillus aculeatus*, the following variants are contemplated, in which at least one of the below-mentioned residues have been amended and/or at least one of the below-mentioned alterations have been introduced::

i) variants of an amended pH-activity profile: D181N;

ii) variants of an amended thermostability, by insertion of prolines: T3P, Y20P, N24P, L25P, T29P, A31P, V50P, S53P, S56P, T93P, T94P, S96P, W142P, L144P, E146P, T147P, T172P, E200P, S203P, A219P, A256P, A258P, S261P, S264P, I266P, T288P, I301P, A304P, Y318P, E324P;

iii) variants of an amended thermostability, by disulfide bridges (double mutations to cysteines): L13C+L65C, N24C+Q30C, S218C+A221C, A304C+Y318C.

Based on the 3D-structure of the galactanase from *Bacillus licheniformis*, the following variants are contemplated, in which at least one of the below-mentioned residues have been amended and/or at least one of the below-mentioned alterations have been introduced::

i) variants of an amended thermostability, by insertion of prolines: K-6P, S-4P, L-2P, K1P, V20P, S26P, K29P, D31P, A54aP, G54eP, N57P, K93P, A97P, N101P, S171P, S185P, T256P, N260P, N266P, D286P, E288aP, A289P, A302dP, S302yP, Y302zP, A302bbP, E302ccP, E302ggP, F305P, D311P, F318P;

ii) variants of an amended thermostability, by disulfide bridges (double mutations to cysteines): S18C+Y302qC, G40C+Q330C, V44C+A69C, I48C+A62C, N50C+D84C,

G54gC+T302xC, N56C+G302rC, A62C+G146C, K106C+A159C, K114C+A163C, E183C+G221C, T227C+A283C, A234C+V241C, Y250C+Q273C, A302aaC+A302iiC.

Additional variants of the invention which may exhibit amended properties as regards substrate binding and/or substrate specificity are listed below.

According to "Nomenclature for sugar-binding subsites in glycosyl hydrolases", G.J. Davis, K.S. Wilson and B. Henrissart, Biochemical Journal, Volume 321, pages 557 to 559 (1997), so-called subsites may be determined. Such subsites may be labelled from -N to +N (where N is an integer). -N represents the non reducing end and +N the reducing end of the polysaccharide. The cleaveage is taking place between the -1 and +1 subsites. The principal constituent of a sugar binding subsite is also called an aromatic platform. That is an aromatic residue, i.e. one of the following: W, H, Y or F.

Based on Figs. 1-4 the inventors identified subsites as follows:

For MTGAL, HIGAI and AAGAL the following subsites were identified, reference being here had to the position numbering of SEQ ID NOs 1, 2, and 3, respectively (not to the corresponding residue in SEQ ID NO: 1):

Subsite -4: MTGAL none; HIGAL W53; AAGAL none.

Subsite -2: MTGAL W86, W300; HIGAL W86, W300; AAGAL W86, W301.

Subsite -1: MTGAL W296; HIGAL W296; AAGAL W297.

Subsite +1: MTGAL Y217, Y214; HIGAL Y217, Y214; AAGAL Y218, Y215.

Subsite +2: MT W183; HIGAL W183; AAGAL W184.

For BLGAL the following subsites were identified, reference being here had to the position numbering of SEQ ID NO: 4 (not to the corresponding residue in SEQ ID NO: 1):

Subsite -4: W363.

Subsite -3: W347.

Subsite -2: W115.

Subsite -1: W320.

Subsite +1: W237, Y234.

Also the residues in the near vicinity (5 Å) of the above residues may be altered and provide an amended substrate specificity and/or substrate binding. These residues are the following, reference being here had to the position numbering of SEQ ID NOs 1, 2, 3, and 4, respectively (not to the corresponding residue in SEQ ID NO: 1):

MTGAL (SEQ ID NO: 1): G6, V7, D8, W9, S10, S11, V12, V13, V14, E15, E16, A18, V20, Y22, L32, L36, T43, V44, R45, Q46, R47, V48, W49, V50, N51, P52, D54, N56, Y57, Y61, Y77, D79, F80, H81, Y82, S83, D84, T85, W86, A87, D88, P89, A90, H91, Q92, T93, M94, P95, G133, N134, E135, I136, R137, G139, L140, L141, W142, H178, L179, D180, N181, G182, W183, D184, W185, G186, T187, Q188, N189, G210, V211, S212, F213, Y214, P215, F216, Y217, S218, S219, S220, A221, T222, L223, S224, A225, L226, K227, S228, S229, L230, D231, N232, M233, I241, A242, V243, V244, E245, T246, N247, W248,

P249, I250, C252, P255, R256, Y257, S258, F259, P260, D262, V263, Q273, F276, I277, V280, I283, L293, F294, Y295, W296, E297, P298, A299, W300, I301, H302, N303, A304, N305, L306, G307, S308, S309, C310, A311, D312, N313, T314, M315, F316, S317, Q318, S319, G320, Q321, L326, F329.

HIGAL (SEQ ID NO: 2): G6, V7, D8, W9, S10, S11, V12, M13, V14, E15, E16, A18, V20, Y22, L32, L36, M43, V44, R45, Q46, R47, V48, W49, V50, N51, P52, W53, D54, G55, N56, Y57, N58, Y61, Y77, N79, F80, H81, Y82, S83, D84, T85, W86, A87, D88, P89, A90, H91, Q92, T93, T94, A96, G133, N134, E135, I136, T137, G139, L141, W142, H178, L179, D180, N181, G182, W183, N184, W185, D186, T187, Q188, N189, G210, V211, S212, F213,

Y214, P215, F216, Y217, S218, A219, S220, A221, T222, L223, D224, S225, L226, R227, R228, S229, L230, N231, N232, M233, V241, A242, V243, V244, E245, T246, N247, W248, P249, C252, P255, R256, Y257, Q258, F259, P260, D262, V263, Q273, Y276, I277, V280, V283, L293, F294, Y295, W296, E297, P298, A299, W300, I301, H302, N303, A304, N305, L306, G307, S308, S309, C310, A311, D312, N313, T314, M315, F316, T317, P318, S319, G320, Q321, L326, F329.

AAGAL (SEQ ID NO: 3): R5, G6, A7, D8, I9, S10, S11, L12, L13, L14, L15, E16, E18, Y20, Y22, L32, L36, S43, I44, R45, Q46, R47, V48, W49, V50, N51, P52, D54, S56, Y57, Y61, Y77, D79, L80, H81, L82, S83, D84, T85, W86, A87, D88, P89, S90, D91, Q92, T93, T94, P95, G134, N135, E136, I137, R138, G140, L142, W143, H179, L180, D181, D182, G183, W184, S185, W186, D187, Q188, Q189, N190, G211, V212, S213, Y214, Y215, P216, F217, Y218, S219, A220, S221, A222, T223, L224, A225, S226, L227, K228, T229, S230, L231, A232, N233, L234, V243, V244, V245, E246, T247, N248, W249, P250, C253, P256, A257, Y258, A259, F260, P261, D263, L264, Q274, F277, L278, L281, V284, V294, Y295, Y296, W297, E298, P299, A300, W301, I302, G303, N304, A305, G306, L307, G308, S309, S310, C311, A312, D313, N314, L315, M316, V317, D318, Y319, T320, D322, V324, Y325, I328, L331.

BLGAL (SEQ ID NO: 4): K26, G27, V28, D29, V30, S31, S32, A35, L36, Y64, V65, R66, V67, R68, I69, W70, N71, D72, P73, Y74, G80, Y81, G82, G83, G84, N85, N86, L106, D108, F109, H110, Y111, S112, D113, F114, W115, A116, D117, P118, A119, K120, Q121, K122, A123, P124, Q161, G163, N164, E165, T166, G169, A171, G172, H202, F203, T204, N205, P206, E207, T208, R211, Y212, S231, S232, Y233, Y234, P235, F236, W237, H238, G239, T240, L241, N243, L244, V261, A262, E263, T264, S265, Y266, T267, D274, G275, H276, G277, N278, T279, A280, P281, K282, N283, G284, Q285, T286, L287, N288, Q296, A299, V300, V303, V317, F318, Y319, W320, E321, P322, A323, W324, I325, V327, N336, K337, L339, W340, E341, Y343, G344, S345, G346, W347, A348, T349, S350, Y351, A352, A353, Y355, D356, P357, E358, D359, A360, G361, K362, W363, F364, G365, G366, S367, A368, V369, D370, N371, Q372, A373, L374, F375, F388.

The above amino acids may be substituted with any other amino acid, e.g. any of the remaining 19 natural amino acids. In the variants of the invention, at least one of the above-mentioned residues have been amended to introduce either of the other nineteen amino acid residues. The above variants are also included in dependent claims, however in the claims they have been renumbered according to the principles outlined above, each position being assigned the number of the corresponding amino acid residue in SEQ ID NO: 1.

Alignments

The program ClustalW (CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice." Julie D. Thompson, Desmond G. Higgins, and Toby J. Gibson, Nucleic Acids Research, 22(22):4673-4680 (1994)) is used for the purposes of the present invention for pairwise protein sequence alignments, multiple protein sequence alignments and protein profile alignments (version 1.82, default parameters).

For pairwise sequence comparison and calculation of percentage identity, the pairwise alignment parameters were: Slow/Accurate; Gap Open Penalty=10.00; Gap Extension Penalty=0.10; Protein weight matrix=Gonnet series; DNA weight matrix=IUB.

The consensus length is calculated automatically by the program. The number of identical residues (identified with an asterisk) is counted. The percentage of sequence identity is calculated as follows: the number of identical residues is divided by the consensus length and multiplied by 100.

The multiple alignment of Fig. 5 is based on a multiple alignment of the four sequences using Clustalw, but, importantly, it is combined with information derived from the 3D-structures, each position in each backbone being carefully evaluated, and the alignment modified by the present inventors. In other words, the multiple alignment of Fig. 5 is not a simple ClustalW multiple alignment reflecting only sequence homologies, it also reflects structural similarities.

The alignment of Fig. 5 can therefore be used to deduce corresponding variants in other backbones, and these variants are likely to also exhibit the amended property in question. For example, the above-mentioned variant A90S+H91D of MT is transferable to the other backbones or parent galactanases shown in Fig. 5 as follows: According to the Fig. 5 alignment, this variant would correspond to: A90S+H91D of HI; and A90S+K91D of BL. Because AA already has the sequence of S90D91, this variant is not relevant for AA. Another example is variant T288P of AA, which, using the alignment of Fig. 5, translates into S288P in MT and HI, and G288P in BL.

Other galactanase backbones of Glycoside Family 53 are known (see below under parents), and these can be added to the alignment of Fig. 5 as described below, and thereby corresponding variants can be deduced also for these backbones, as just described above.

For aligning a new sequence to the multiple alignment of Fig. 5, the Clustalw option called profile alignment is used as follows: The Fig. 5 multiple alignment is used as profile 1, and then the new sequence as profile 2. Then the program is asked to "Align sequence to 1st. profile," using the following parameters:

Multiple alignment parameters = Slow/Accurate; Gap Open Penalty=10.00; Gap Extension Penalty=0.20; Delay divergent sequences=30%; DNA Transitions Weight:0.50; Protein weight matrix=Gonnet series; DNA weight matrix=IUB; Use negative matrix=OFF;

Protein Gap Parameters: Toggle Residue-Specific Penalties=ON; Toggle Hydrophilic Penalties=ON; Hydrophilic Residues= GPSNDQEKR; Gap Separation Distance =4; Toggle End Gap Separation=OFF.

In Fig. 6, as an example, three new galactanase sequences have been added to the Fig. 5 alignment. The new galactanases are added at the bottom of the alignment, as rows nos. 5, 6 and 7. The galactanases are: AT (the galactanase of *Aspergillus tubigensis*, (SEQ ID NO: 7)); BS (the galactanase of *Bacillus subtilis* (SEQ ID NO: 8)); and PF (the galactanase of *Pseudomonas fluorescens* (SEQ ID NO: 9)). Thus, using Fig. 6, the above-mentioned variant A90S+H91D of MT translates into A90S+K91D of BS, and E90S+K91D of PF. Because AT already has the sequence of S90D91, this variant is not relevant for AT. Another example is variant T288P of AA, which, using the alignment of Fig. 6, translates into variants T288P of AT, G288P of BS, and G288P of PF.

In the alternative, alignments of sequences and calculation of degree %-identity may be done using a full Smith-Waterman alignment, useful for both protein and DNA alignments. The default scoring matrices BLOSUM50 and the identity matrix are used for protein and DNA alignments respectively. The penalty for the first residue in a gap is -12 for proteins and -16 for DNA, while the penalty for additional residues in a gap is -2 for proteins and -4 for DNA. Alignment may be made with the FASTA package version v20u6 (W. R. Pearson and D. J. Lipman (1988), "Improved Tools for Biological Sequence Analysis", PNAS 85:2444-2448, and W. R. Pearson (1990) "Rapid and Sensitive Sequence Comparison with FASTP and FASTA", Methods in Enzymology, 183:63-98).

Parent

The term "parent galactanase," or simply "parent," refers to the galactanase on which the variant was based, and also to the galactanase with which the variant is compared and aligned.

The parent may be a naturally occurring (wildtype) galactanase, or it may in turn even be a variant thereof, prepared by any suitable means. For instance, the parent galactanase may be a variant of a naturally occurring galactanase which has been modified or altered in the amino acid sequence. A parent may also be an allelic variant which is any of two or more alternative forms of a gene occupying the same chromosomal locus. Allelic variation arises

naturally through mutation, and may result in polymorphism within populations as is well-described in the art. An allelic variant of a polypeptide is a polypeptide encoded by the corresponding allelic variant of a gene.

Galactanase

This section is applicable to the parent galactanases, as well as the variant galactanases of the invention.

Galactanases catalyze the endohydrolysis of 1,4-beta-D-galactosidic linkages in arabinogalactans of type I and/or galactans (see the structure of rhamnogalacturonan I as described in Carpita et al. in Plant J:, 3:1-30, 1993).

In the present context, a galactanase is a polypeptide having galactanase activity. Galactanase activity can be measured using a substrate including 1,4-beta-D-galactosidic linkages. Examples of galactanase substrates are arabinogalactans of type I and galactans. Particularly suitable substrates are i) lupin galactan, and potato galactan (commercially available from, e.g., MegaZyme, Australia); as well as ii) AZCL-galactan substrates such as AZCL-potato-galactan, and AZCL-lupin-galactan (also commercially available from MegaZyme, Australia). For the substrates mentioned under i) above, galactanase activity may be measured as release of reducing sugars, whereas for the AZCL-substrates, the galactanase activity is measured spectrophotometrically (formation of a blue colour). In a particular embodiment, the galactanase assay is based on the substrate lupin AZCL galactan.

The person skilled in the art will know how to adapt assay-pH and assay-temperature to the galactanase in question. Examples of assay-pH-values are pH 2, 3, 4, 5, 6, 7, 8, 9, 10, or 11. Examples of assay-temperatures are 20, 25, 30, 35, 37, 40, 45, 50, 55, 60, 65, 70, 75, 80, or 90°C.

A preferred galactanase assay is described in Example 2 herein.

In a particular embodiment, the galactanase is an enzyme classified as EC 3.2.1.89, the official name of which is arabinogalactan-endo-1,4-beta-galactosidase. Alternative names are endo-1,4-beta-galactanase, galactanase, or arabinogalactanase. EC refers to Enzyme Class as described at a) http://www.chem.qmul.ac.uk/iubmb/enzyme/, and/or in b) Enzyme Nomenclature 1992 from NC-IUBMB, Academic Press, San Diego, California, published by Academic Press for IUBMB in 1992 (ISBN 0-12-227164-5), as regularly supplemented and updated. For supplements and updates, please consult http://www.chem.qmul.ac.uk/iubmb/enzyme/supplements/, giving details regarding the following supplements: Supplement 1 (1993) (Eur. J. Biochem., 1994 223, 1-5) ; Supplement 2 (1994) (Eur. J. Biochem., 1995 232, 1-6) ; Supplement 3 (1995) (Eur. J. Biochem., 1996 237, 1-5) ; Supplement 4 (1997) (Eur. J. Biochem., 1997, 250, 1-6) ; Supplement 5 (1999)

(Eur. J. Biochem., 1999, 264, 610-650) : Supplement 6 (2000) ; Supplement 7 (2001); and Supplement 8 (2002).

Glycoside Hydrolase (GH) Family 53

The EC-classification referred to above is mainly based on substrate specificity of the enzymes, and does therefore not reflect the structural features of these enzymes. A classification of glycoside hydrolases in families based on amino acid sequence similarities has been proposed a few years ago; see the CAZy(ModO) site at the internet:

Coutinho, P.M. & Henrissat, B. (1999) Carbohydrate-Active Enzymes server at URL: http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html; and/or Coutinho, P.M. & Henrissat, B. (1999) Carbohydrate-active enzymes: an integrated database approach. In "Recent Advances in Carbohydrate Bioengineering", H.J. Gilbert, G. Davies, B. Henrissat and B. Svensson eds., The Royal Society of Chemistry, Cambridge, pp. 3-12; Coutinho, P.M. & Henrissat, B. (1999) The modular structure of cellulases and other carbohydrate-active enzymes: an integrated database approach. In "Genetics, Biochemistry and Ecology of Cellulose Degradation"., K. Ohmiya, K. Hayashi, K. Sakka, Y. Kobayashi, S. Karita and T. Kimura eds., Uni Publishers Co., Tokyo, pp. 15-23; Henrissat B., A classification of glycosyl hydrolases based on amino-acid sequence similarities. Biochem. J. 280:309-316(1991); Henrissat B., Bairoch A. New families in the classification of glycosyl hydrolases based on amino- acid sequence similarities. Biochem. J. 293:781-788(1993); Henrissat B., Bairoch A. Updating the sequence-based classification of glycosyl hydrolases. Biochem. J. 316:695-696(1996); and/or Davies G., Henrissat B. Structures and mechanisms of glycosyl hydrolases. Structure 3:853-859(1995).

Glycoside Hydrolase Family 53 is found under the entry relating to Glycosidases and Transglycosidases (or Glycoside Hydrolases).

These are particular embodiments of the GH Family 53 galactanase,

i) it is an endo-1,4-beta-galactanase (EC 3.2.1.89);

ii) it has a retaining catalytic mechanism;

iii) it has Glu as a catalytic nucleophile or base;

iv) it has Glu as a catalytic proton donor;

v) its 3D Structure has a fold $(beta/alpha)_8$; and/or

vi) it belongs to GH Clan GH-A .

For the purposes of the present invention, the below glycoside hydrolases of Family 53 are non-limiting examples of a parent galactanase:

Protein	Organism	GenBank	GenPept	SwissProt	Publication
galactanase 1	*Aspergillus aculeatus*	L34599	AAA32692.1	P48842	Christgau et al, Curr. Genet. 27:135-

					141(1995)
endo-1,4-beta-galactanase (GalA)	*Aspergillus niger*	AJ305303	CAC83735.1	Q8X168	-
galactanase GalA	*Aspergillus tubingensis*	AJ012316	CAB40555.1	Q9Y7F8	Van der Vlugt-Bergmans et al, Biotechnol. Tech. 13:87-92(1999)
ORF 1	*Bacillus circulans*	L03425	AAA22259.1	P48843	SEQ ID NO:10 of WO 00/47711
ORF BH2023	*Bacillus halodurans*	AP001514 NC_002570	BAB05742.1 NP_242889.1	Q9KBA5	Takami et al, Extremophiles 3 (1), 21-28 (1999)
ORF yvfO	*Bacillus subtilis*	Z94043 Z99121	CAB08009.1 CAB15417.1	O07013 O07013 O32260	SEQ ID NO: 14 of WO 00/47711
YvfO	*Bifidobacterium longum*	AE014643 NC_004307	AAN24099.1 NP_695463.1		Schell et al, Proc. Natl. Acad. Sci. U.S.A. 99 (22), 14422-14427 (2002)
galactanase	*Cellvibrio japonicus* (*Pseudomonas cellulosa*)	X91885	CAA62990.1	P48841	Braithwaite et al, Biochemistry 36:15489-15500 (1997)
ORF CAC2570	*Clostridium acetobutylicum*	AE007755	AAK80519.1	Q97G04	Nolling et al, J. Bacteriol. 183 (16), 4823-4838 (2001)
ORF TM1201	*Thermotoga maritima*	AE001777 NC_000853	AAD36276.1 NP_229006.1	Q9X0S8	Nelson et al, Nature 399:323-329(1999)
Sequence 2 from patent US 6242237	*Myceliophthora thermophila*	AAE73520	AAE73520.1		US 6242237
Sequence 4 from patent US 6242237	*Humicola insolens*	AAE73521	AAE73521.1		US 6242237
ORF GalA	*Xanthomonas axonopodis pv.*	AE011762 NC_003919	AAM36180.1 NP_641644.1		da Silva et al, Nature 417 (6887), 459-

	citri				463 (2002)
ORF XAC0575	*Xanthomonas axonopodis pv. citri*	AE011684 NC_003919	AAM35464.1 NP_640928.1		da Silva et al, Nature 417 (6887), 459-463 (2002)
ORF GalA	*Xanthomonas campestris pv. campestris*	AE012224 NC_003902	AAM40555.1 NP_636631.1		da Silva et al, Nature 417 (6887), 459-463 (2002)
ORF GalA	*Xanthomonas campestris pv. campestris*	AE012483 NC_003902	AAM42894.1 NP_638970.1		da Silva et al, Nature 417 (6887), 459-463 (2002)
ORF YPO0853	*Yersinia pestis*	AJ414145 NC_003143	CAC89700.1 NP_404474.1	Q8ZHN7	Parkhill et al, Nature 413:523-527(2001)
ORF Y3238	*Yersinia pestis*	AE013925 NC_004088	AAM86788.1 NP_670537.1		Deng et al J. Bacteriol. 184 (16), 4601-4611 (2002)

Additional examples of a parent galactanase of the invention are the galactanases derived from *Meripilus giganteus* (SEQ ID NO: 2 of WO 97/32013), *Pseudomonas fluorescens*, *Bacillus agaradhaerens* (SEQ ID NO: 12 of WO 00/47711), and *Bacillus licheniformis* (SEQ ID NO: 8 of WO 00/47711).

The present invention specifically includes variants of each and every of the above specific parent galactanases of GH Family 53 corresponding to the claimed variants of MTGAL, HIGAL, AAGAL and BLGAL, such variants being derivable by adding the parent galactanase sequence in question to the Fig. 5 alignment as described above for the construction of Fig. 6, and translating each MTGAL, HIGAL, AAGAL, or BLGAL variant into the parent galactanase in question, using the concept of corresponding amino acid residue as defined above.

In a first embodiment, the parent GH Family 53 galactanase is a fungal galactanase. The fungal galactanase may be derived from a yeast, or from a filamentous fungus. The yeast galactanase may be derived from *Yersinia*, e.g. from *Yersinia pestis*. The filamentous fungal galactanase may be derived from a strain of *Aspergillus*, *Humicola*, *Meripilus*, *Myceliophthora*, or *Thermomyces*. Examples of these strains are *Aspergillus aculeatus*, *Aspergillus niger*, *Aspergillus tubingensis*, *Humicola insolens*, *Meripilus giganteus*, and *Myceliophthora thermophila*.

In a second embodiment, the parent GH Family 53 galactanase is a bacterial galactanase. The bacterial galactanase may be derived from a strain of *Bacillus*, *Bifidobacterium*, *Cellvibrio*, *Clostridium*, *Pseudomonas*, *Thermotoga*, or *Xanthomonas*.

Examples of such strains are *Bacillus agaradhaerens*, *Bacillus circulans*, *Bacillus halodurans*, *Bacillus licheniformis*, *Bacillus subtilis*, *Bifidobacterium longum*, *Cellvibrio japonicus*, *Clostridium acetobutylicum*, *Pseudomonas fluorescens*, *Pseudomonas cellulosa*, *Thermotoga maritime*, *Xanthomonas axonopodis* pv. *citri*, and *Xanthomonas campestris pv. campestris*.

Particularly preferred parent galactanases are those with the above-mentioned GenBank, GenPept, or SwissProt accession numbers, and those with the above-mentioned SEQ ID NO's.

Further particularly preferred GH Family 53 parent galactanases are the following:

Strain of origin	Sequence Number (herein)	Abbreviations used herein
Myceliophthora thermophila	SEQ ID NO: 1	MTGAL, or MT
Humicola insolens	SEQ ID NO: 2	HIGAL, or HI
Aspergillus aculeatus	SEQ ID NO: 3	AAGAL, or AA
Bacillus licheniformis	SEQ ID NO: 4	BLGAL, or BL

Preferred subgroups of the above are a) MTGAL, HIGAL, AAGAL; b) MTGAL, HIGAL, BLGAL; and c) MTGAL, HIGAL.

In a third embodiment, the parent galactanase has a percentage identity to SEQ ID NO: 1 of at least 25%, using the program ClustalW and the settings referred to above. In further particular embodiments, the percentage identity is at least 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, or at least 95%.

In a fourth embodiment, the galactanase variant has a percentage identity to SEQ ID NO: 1 of at least 50%, using the program ClustalW and the settings referred to above. In further particular embodiments, the percentage identity is at least 55, 60, 65, 70, 75, 80, 85, 90, 95, 97, or at least 99%.

In a fifth embodiment, the parent galactanase has a percentage identity to SEQ ID NO: 2 of at least 25%, using the program ClustalW and the settings referred to above. In further particular embodiments, the percentage identity is at least 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, or at least 95%.

In a sixth embodiment, the galactanase variant has a percentage identity to SEQ ID NO: 2 of at least 50%, using the program ClustalW and the settings referred to above. In further particular embodiments, the percentage identity is at least 55, 60, 65, 70, 75, 80, 85, 90, 95, 97, or at least 99%.

In a seventh embodiment, the parent galactanase has a percentage identity to SEQ ID NO: 3 of at least 25%, using the program ClustalW and the settings referred to above. In further particular embodiments, the percentage identity is at least 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, or at least 95%.

In an eighth embodiment, the galactanase variant has a percentage identity to SEQ ID NO: 3 of at least 50%, using the program ClustalW and the settings referred to above. In

further particular embodiments, the percentage identity is at least 55, 60, 65, 70, 75, 80, 85, 90, 95, 97, or at least 99%.

In a ninth embodiment, the parent galactanase has a percentage identity to SEQ ID NO: 4 of at least 25%, using the program ClustalW and the settings referred to above. In further particular embodiments, the percentage identity is at least 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, or at least 95%.

In a tenth embodiment, the galactanase variant has a percentage identity to SEQ ID NO: 4 of at least 50%, using the program ClustalW and the settings referred to above. In further particular embodiments, the percentage identity is at least 55, 60, 65, 70, 75, 80, 85, 90, 95, 97, or at least 99%.

In further particular embodiments of each of the above first to tenth embodiments, the alignment is a full Smith-Waterman alignment with the settings referred to above, preferably made with the FASTA package also referred to above.

It is to be understood that also variants of galactanases are contemplated as the parent enzyme.

Preparation of galactanase variants

The galactanase variants may be prepared by any method known in the art, see e.g. Example 1 herein. Typically, a galactanase variant library is prepared. The term "randomized library", "variant library", or simply "library" refers to such library of galactanase variants. Diversity in the variant library can be generated via mutagenesis of the genes encoding the variants at the DNA triplet level, such that individual codons are variegated e.g. by using primers of partially randomized sequence in a PCR reaction. Several techniques have been described, by which one can create a diverse combinatorial library by variegating several nucleotide positions in a gene and recombining them, for instance where these positions are too far apart to be covered by a single (spiked or doped) oligonucleotide primer. These techniques include the use of in vivo recombination of the individually diversified gene segments as described in WO 97/07205 on page 3, lines 8 to 29 (Novozymes A/S). They also include the use of DNA shuffling techniques to create a library of full length genes, wherein several gene segments are combined, and wherein each segment may be diversified e.g. by spiked mutagenesis (Stemmer, Nature 370, pp. 389-391, 1994 and US 5,811,238; US 5,605,793; and US 5,830,721). One can use a gene encoding a galactanase "backbone" (wildtype parent galactanase) as a template polynucleotide, and combine this with one or more single or double-stranded oligonucleotides as described in WO 98/41623 and in WO 98/41622 (Novozymes A/S). The single-stranded oligonucleotides could be partially randomized during synthesis. The double-stranded oligonucleotides could be PCR products incorporating diversity in a specific region. In both cases, one can dilute the

diversity with corresponding segments encoding the sequence of the backbone galactanase in order to limit the average number of changes that are introduced.

Methods have also been established for designing the ratios of nucleotide mixtures (A; C; T; G) to be inserted in specific codon positions during oligo- or polynucleotide synthesis, so as to introduce a bias in order to approximate a desired frequency distribution towards a set of one or more desired amino acids that will be encoded by the particular codons. It may be of interest to produce a variant library that comprises permutations of a number of known amino acid modifications in different locations in the primary sequence of the polypeptide. These could be introduced post-translationally or by chemical modification sites, or they could be introduced through mutations in the encoding genes. The modifications by themselves may previously have been proven beneficial for one reason or another (e.g. decreasing antigenicity, or improving specific activity, performance, stability, or other characteristics). In such instances, it may be desirable first to create a library of diverse combinations of known sequences. For example, if twelve individual mutations are known, one could combine (at least) twelwe segments of the parent protein encoding gene, wherein each segment is present in two forms: one with, and one without the desired mutation. By varying the relative amounts of those segments, one could design a library (of size 212) for which the average number of mutations per gene can be predicted. This can be a useful way of combining mutations, that by themselves give some, but not sufficient effect, without resorting to very large libraries, as is often the case when using 'spiked mutagenesis'. Another way to combine these 'known mutations' could be by using family shuffling of oligomeric DNA encoding the known mutations with fragments of the full length wild type sequence.

The mutated DNA can be expressed by any method known in the art, see e.g. Example 1. Generally, the host cell may be a unicellular microorganism, e.g., a prokaryote, or a non-unicellular microorganism, e.g., a eukaryote.

Useful unicellular cells are bacteria such as *Bacillus*, *Streptomyces*, *E. coli*, *Pseudomonas* sp., *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Streptococcus*, *Pediococcus*, and *Enterococcus*.

Examples of eukaryote cells are non-human animal cells, insect cells, plant cells, or fungal cells. Examples of fungal cells are *Candida*, *Hansenula*, *Kluyveromyces*, *Pichia*, *Saccharomyces*, *Schizosaccharomyces*, *Yarrowia*, *Acremonium*, *Aspergillus*, *Fusarium*, *Humicola*, *Mucor*, *Myceliophthora*, *Neurospora*, *Penicillium*, *Thielavia*, *Tolypocladium*, and *Trichoderma*.

Applications

The galactanase variants of the invention are useful in animal feed, see e.g. WO 97/16982. Non-limiting examples of desirable characteristics of galactanase variants for feed applications are: High temperature stability, acid-stability and high specific activity.

The galactanase variants of the invention, e.g., but not exclusively, those of claims 1-4, may also be used to prepare galacto-oligo-saccharides and for hydrolysis of lactose, both of which are relevant for, e.g., the dairy industry. For example, the method of Example 5 can be used for screening of galactanase variants for improved activity on lactose, in particular for improved transglycosylation and/or hydrolytic activity on lactose.

The transglycosylation reactions observed with ONPG (Example 4) can be used for screening of galactanase variants for suitable acceptor affinities. The screening may be a high-through-put screening. This provides valuable knowledge of the affinities of the individual subsites (such as subsites +1, +2, +3, +4) for various acceptors, e.g. galactose (Gal), ß-1,4-galactobiose (Gal2) (Megazyme), ß-1,4-galactotriose (Gal3), ß-1,4-galactotetraose (Gal4), glucose (Glu), arabinose (Ara), galacturonic acid (GalA), maltose (Mal) or maltotriose (Mal3).

The results of Example 3 provides knowledge of individual subsites for galactose (-3 to +3), as well as knowledge of the tendencies to transglycosylate instead of hydrolyse substrates. This knowledge is useful for the designing of galactanase variants of desired properties.

Various references are cited herein, the disclosures of which are incorporated by reference in their entireties.

EXAMPLES

Example 1: Preparation of galactanase variants

The D181N mutation was introduced in the AAGAL encoding gene by the use of the mutagenic oligonucleotide 5'- CAT TTG GAC AAC GGC TGG AGC -3' (SEQ ID NO: 5) and the mega-priming method described by Sarkar, G., and Sommer, S.S., 1990. The "Megaprimer" Method of Site-Directed Mutagenesis. BioTechniques, 8: 404-407. The mutations D181N+S90A+D91H were introduced in a similar way.

The resulting variant genes were cloned into plasmid pHD464 as desribed in Dalbøge H., Heldt-Hansen H. 1994. A novel method for efficient expression cloning of fungal enzyme genes. Mol. Gen. Genet. 243: 253-260, and the correct introduction of the mutations were verified by DNA sequencing.

The A90S+H91D double mutation was introduced in the MTGAL encoding gene essentially as described above by the use of the mutageneic oligonucleotode 5'- GCC GAT CCT TCT GAT CAG ACC ATG CC -3' (SEQ ID NO: 6).

Proteins were expressed in, and secreted from *Aspergillus oryzae* essentially as described in Christensen, T., Wöldike, H., Boel, E., Mortensen, S.B., Hjortshøj, K., Thim, L., Hansen, M.T., 1988. High level expression of recombinant genes in Aspergillus oryzae. Bio/Technology 6, 1419-1422.

Example 2: Purification and characterization of galactanase variants

Purification of *Aspergillus aculeatus* galactanase variants

The culture supernatant from a fermentation of the *Aspergillus oryzae* strain expressing the site-directed recombinant *Aspergillus aculeatus* galactanase variant D181N (described in Example 1) was filtered through a 0.22 µm filter to remove the mycelia. 1200 ml filtrate was added ammonium sulphate to a concentration of 1.6 M, loaded onto a 50 ml butyl column equilibrated with 25 mM sodium acetate, 1.6 M ammonium sulphate pH 5.0 and eluted using a linear ammonium sulphate decreasing from 1.6 M to 0 M over 10 column volumes. Galactanase activity was measured by mixing 40 µl of fractions with 200 µl 10 mg/ml lupin AZCL-galactan (Megazyme, Australia) in 0.5 M MES pH 6.5 After about 30 min incubation at room temperature, insoluble substrate was removed by centrifugation, and absorbance of supernatant measured at 590 nm. Fractions containing galactanase activity eluted around 1 M ammonium sulphate were pooled and dialysed against 10 mM sodium citrate pH 3.5. Dialysate (400 ml) was diluted to 2000 ml with water and loaded onto a 50 ml S-Sepharose column equilibrated with 10 mM sodium citrate pH 3.5. Galactanase activity did not bind to this column and was concentrated to 80 ml on an Amicon ultrafiltration device with a 10 kDa cut off filter. The concentrate was at least 95% pure estimated from SDS-PAGE.

The culture supernatant from a fermentation of the *Aspergillus oryzae* strain expressing the site-directed recombinant *Aspergillus aculeatus* galactanase variant D181N+S90A+D91H was filtered as described above. 900 ml filtrate was added ammonium sulphate to a concentration of 1.6 M, and eluted from a 50 ml butyl column as described above. Galactanase activity was measured as described above. Fractions containing galactanase activity eluted around 0.35 M ammonium sulphate and were pooled and dialysed against 25 mM sodium acetate pH 5.5. Dialysate (200 ml) was diluted to 275 ml with water, loaded onto a 50 ml Q-Sepharose column equilibrated with 25 mM sodium acetate pH 5.5, and eluted with a linear gradient from 0 to 1 M NaCl over 10 column volumes. Fractions containing galactanase activity (around 0.8 M NaCl) were pooled and concentrated to 10 ml on an Amicon ultrafiltration device with a 10 kDa cut off filter. The concentrate was at least 95% pure estimated from SDS-PAGE.

Purification of *Myceliophthora thermophila* galactanase variants

The culture supernatant from a fermentation of the *Aspergillus oryzae* strain expressing the site-directed recombinant *Myceliophthora thermophila* galactanase variant A90S+H91D (described in Example 1) was filtered through a 0.22 µm filter to remove the mycelia. 1200 ml filtrate was added ammonium sulphate to a concentration of 1.6 M, loaded onto a 50 ml butyl column equilibrated with 25 mM sodium acetate, 1.6 M ammonium sulphate pH 5.0 and eluted using a linear ammonium sulphate decreasing from 1.6 M to 0 M over 10 column volumes. Galactanase activity was measured by mixing 40 µl of fractions with 200 µl 10 mg/ml lupin AZCL-galactan (Megazyme, Australia) in 0.5 M MES pH 6.5 After about 30 min incubation at room temperature, insoluble substrate was removed by centrifugation, and absorbance of supernatant measured at 590 nm. Fractions containing galactanase activity eluted around 1 M ammonium sulphate were pooled and dialysed against 10 mM sodium citrate pH 3.5. Dialysate (400 ml) was diluted to 2000 ml with water and loaded onto a 50 ml S-Sepharose column equilibrated with 10 mM sodium citrate pH 3.5. Galactanase activity did not bind to this column and was concentrated to 80 ml on an Amicon ultrafiltration device with a 10 kDa cut off filter. The concentrate was at least 95% pure estimated from SDS-PAGE.

Characterization of the purified variants

The pH profiles of the purified variants described above were established as follows: Galactanase activity at various pH was measured by mixing 500 µl 4 mg/ml lupin AZCL-galactan (Megazyme, Australia) in water with 500 µl buffer (50 mM sodium acetate, 50 mM potassium dihydrogenphosphate, 50 mM boric acid, 1 mM $CaCl_2$, 0.01% Triton X-100 adjusted to pH 2.5, 3.5, 4.5, 5.5, 6.5, 7.5, 8.5 or 9.5 with HCl/NaOH) and 25 µl purified enzyme diluted to about 0.5-2 µg/ml in water. The mixture was incubated 15 min at 37°C, insoluble material was removed by centrifugation, and absorbance in the supernatant was measured at 590 nm.

From the results shown in Table 1 below, it appears that the pH profiles have changed (the profile of the AAGAL variants D181N, and D181N+S90A+D91H have been shifted to the alkaline side; and the pH profile of the MTGAL variant A90S+H91D has been shifted to the acidic side, as compared to the wild types).

Table 1

Galactanase / pH	2.5	3.5	4.5	5.5	6.5	7.5	8.5	9.5
AAGAL	73	100	83	47	32	0	2	0
AAGAL D181N	74	99	100	87	74	35	7	0
AAGAL D181N+S90A+D91H	55	59	71	83	100	90	21	0
MTGAL	0	12	41	63	90	100	54	7
MTGAL A90S+H91D	0	8	51	75	100	95	35	4

Example 3: Activity on galactooligosaccharides

Preparation of galactotriose (Gal3), galactotetraose (Gal4), methyl-galactotrioside (MeGal3) and methyl-galactotetraoside (MeGal4)

Galactan (lupin) was purchased from Megazyme. All solvents, reagents and TLC-plates (Silica gel 60 F_{254}) were purchased from Merck. ^{1}H NMR spectra were recorded on a Varian Mercury 400 MHz at 30°C. As reference values $CHCl_3$ in $CDCl_3$ (7.27 ppm) and HDO in D_2O (4.67 ppm) were used. Flash chromatography was accomplished using a FLASH 40i chromatography module from Biotage.

Undeca-O-acetyl galactotriose: Arabinofuranosidase treated lupin galactan (0.50 g) was dissolved in 10 mM Bis-Tris buffer pH 6.5 (50 mL) by stirring for 1 h at 37°C. BLGAL was added (250 GalU/mL) and the solution stirred for 3 h at 37°C and then 5 min at 100°C. TLC (eluent: propanol/ethanol/H_2O (7:1:2)) showed a major (Gal3) and a minor product (Gal4) both eluting below commercial galactobiose. After cooling, the solution was concentrated, dried and acetylated and worked up by standard procedures (Ac_2O/pyridine, 48 h at room temperature (rt)). The crude product was purified by flash chromatography (eluent: EtOAc/heptane 5:2) to give 0.20 g of pure Gal3 peracetate (mixture of alpha- and beta-anomer (1:2)). ^{1}H NMR (selected data, $CDCl_3$): 6.29 ppm (d, $J_{1,2}$ = 3.5 Hz, H-1alpha), 5.63 ppm (d, $J_{1,2}$ = 8.4 Hz, H-1ß).

Galactotriose (Gal3): Deacetylation of the acetylated triose was accomplished by stirring overnight in methanol/$NaOCH_3$ (1 mL 1M $NaOCH_3$ in 3 mL methanol) and then neutralized by addition of Dowex 50 Wx8. Water (2 mL) was added and the resin removed by filtration. The clear solution was concentrated (freeze-drying) to give 0.10 g of solid G3. MS (MALDI-TOF): 527 (M+23, Na). ^{1}H NMR (selected data, D_2O): 5.20 ppm (d, J = 3.6 Hz, H-1alpha), 4.5-4.6 (3 x d, H-1ß, H-1', H-1'').

Methyl deca-O-acetyl galactotrioside: The acetylated galactotriose (0.24 g) was converted into the bromide by treatment (5 h) with 30% HBr in acetic acid (2.5 mL) and CH_2Cl_2 (2 mL) at 0°C → rt. The reaction was worked up by standard procedures and concentrated to give a yellowish syrup (194 mg) of the alpha-bromo compound, which was used without further purification. ^{1}H NMR (selected data, $CDCl_3$): 6.57 ppm (d, 1H, $J_{1,2}$ = 3.8 Hz, H-1). The bromo-glycoside (0.19 g, 0.20 mmol) was converted into the methyl glycoside by overnight

treatment with Ag_2CO_3 (60 mg, 22 mmol) in dry methanol (10 mL) (under nitrogen). After work up, the methyl glycoside was purified by flash chromatography (eluent: EtOAc/heptane (3:1)) to give 30 mg of pure compound (colorless oil). ^1H NMR (selected data, $CDCl_3$): 4.48 ppm, 4.39 ppm and 4.35 ppm (3xd, 3H, $J_{1,2}$ = 8.0 Hz, H-1, H-1' and H-1''), 3.47 ppm (3H, s, OCH_3).

Methyl galactotrioside (MeGal3): The acetylated methyl glycoside (30 mg) was deacetylated as described above to give 10 mg of syrupy material.

Galactotetraose (Gal4): This was prepared as described for Gal3 using 100 GalU/mL. Yield of final deacetylated product: 17 mg.

Methyl galactotetraoside (MeGal4): This compound was prepared in analogy with MeGal3 and 41 mg of MeGal4 was obtained from 1 g of galactan. MS (MALDI-TOF): 704 (M+23, Na).

Activity of HIGAL, MTGAL, AAGAL and BLGAL on galactooligosaccharides

The activity on the galacto-oligosaccharide substrates prepared as described above and on the commercially available galactobiose (Gal2, Megazyme) was studied for the four purified galactanases HIGAL, MTGAL, AAGAL and BLGAL. The buffers and temperatures used were: 25 mM sodium acetate, 0.5 mM $CaCl_2$, 0.005 % Triton X-100, pH 6.5 at 37°C for HIGAL and MTGAL, 50 mM sodium acetate, 1 mM $CaCl_2$, pH 4 at 30°C for AAGAL and 50 mM Mes, 1 mM $CaCl_2$, pH 6.5 at 30°C for BLGAL. Enzyme concentrations used were 0.8 µg/ml for HIGAL, 0.2 µg/ml for MTGAL, and 10 µg/ml for AAGAL and BLGAL. With HIGAL and MTGAL substrate concentrations were all 0.25 mg/ml, whereas 0.34 mg/ml Gal2, 0.050 mg/ml Gal3 and 0.067 mg/ml Gal4 were used for AAGAL and BLGAL. Enzyme activity in samples withdrawn after various incubation times was inactivated by heating to 95°C for 10 min. Compositions of reaction products were analysed using HPAE-PAD (Dionex) applying a PA-100 column and a linear gradient of sodium acetate (0-0.18 M) in 0.15 M NaOH. Response factors of the individual carbohydrates were estimated from reference runs with MeGal3, MeGal4, Gal, Gal2, Gal3 and Gal4. Selected results are shown in Tables 2-8 below (the figures indicating weight percentage of glactooligosaccharides).

Neither of the enzymes HIGAL, MTGAL, AAGAL or BLGAL had any detectable activity on Gal2 in 24 hours. HIGAL, MTGAL and AAGAL degraded Gal3 to Gal2 and Gal, whereas BLGAL had no visible activity on Gal3 after 24 hours. Incubation of HIGAL and MTGAL with MeGal3 (See Tables 2 and 3) gave much higher release of MeGal than MeGal2, indicating that Gal is released from the reducing end of Gal3 with both enzymes.

HIGAL and MTGAL degraded Gal4 (also containing about 40% Gal3) (Tables 4 and 5) mainly to Gal and Gal2, whereas Gal3 did not accumulate. Results for HIGAL and MTGAL with MeGal4 (Tables 6 and 7) gave initial release mainly of MeGal, MeGal2 and Gal3 and some Gal2 but little Gal, again indicating that Gal is released mainly from the reducing end of Gal4. The production of Gal from MeGal4 in the later stages of the hydrolysis may be mainly due to hydrolysis of transglycosylation products with no methyl group at the reducing end. BLGAL degrades galactotetraose mainly to galactose and galactotriose. With MeGal4 the main products from BLGAL were MeGal and Gal3, indicating that Gal is released from reducing end of Gal4. With AAGAL the initial products from galactotetraose are about equimolar amounts of galactose, galactobiose and galactotriose, but subsequently the galactotriose is degraded to galactobiose and galactose.

Table 2: Degradation of MeGal3 with HIGAL

Incubation time (hours)	**0.0**	**0.5**	**2.2**	**5.0**	**72.0**
Gal	0.0	0.0	2.3	3.0	17.1
Gal2	0.0	3.9	12.5	20.2	36.2
Gal3	0.0	1.2	3.3	10.6	8.0
MeGal	0.0	11.4	18.4	36.8	34.6
MeGal2	0.0	3.3	3.7	5.2	4.2
MeGal3	100.0	80.3	59.7	24.2	0.0

Table 3: Degradation of MeGal3 with MTGAL

Incubation time (hours)	**0.0**	**0.5**	**2.2**	**5.0**	**72.0**
Gal	0.0	0.0	14.1	2.2	6.5
Gal2	0.0	0.0	8.5	10.9	37.2
Gal3	0.0	0.0	0.4	15.7	23.2
MeGal	0.0	10.1	27.6	17.4	28.3
MeGal2	0.0	2.7	1.9	3.2	3.5
MeGal3	100.0	87.2	47.5	50.6	1.3

Table 4: Degradation of Gal4 with HIGAL

Incubation time (hours)	**0.0**	**0.5**	**2.2**	**5.0**	**72.0**
Gal	0.0	5.8	16.7	35.6	65.2
Gal2	0.0	8.1	21.9	34.8	33.6

Gal3	42.0	43.2	39.8	23.9	0.9
Gal4	58.0	42.9	21.6	5.7	0.2

Table 5: Degradation of Gal4 with MTGAL

Incubation time (hours)	**0.0**	**0.5**	**2.2**	**5.0**	**72.0**
Gal	0.0	11.6	14.9	29.2	54.9
Gal2	0.0	9.9	17.4	29.1	43.5
Gal3	42.0	27.7	45.5	29.5	1.5
Gal4	58.0	50.8	22.3	12.1	0.0

Table 6: Degradation of MeGal4 with HIGAL

Incubation time (hours)	**0.0**	**0.5**	**2.0**	**5.0**	**24.0**
Gal	0.0	2.3	1.6	7.4	26.4
Gal2	0.0	6.3	5.0	13.8	25.3
Gal3	0.0	20.6	16.0	19.7	9.1
Gal4	0.0	3.3	3.2	3.2	1.7
MeGal	1.6	12.1	10.5	16.6	19.1
MeGal2	4.7	12.6	13.2	16.1	13.4
MeGal3	14.8	17.2	18.4	15.5	5.0
MeGal4	79.0	25.5	32.1	7.6	0.0

Table 7: Degradation of MeGal4 with MTGAL

Incubation time (hours)	**0.0**	**0.5**	**2.0**	**5.0**	**24.0**
Gal	0.0	0.9	4.8	12.4	24.2
Gal2	0.0	3.3	10.9	20.1	32.7
Gal3	0.0	13.7	23.9	17.9	3.4
Gal4	0.0	2.5	3.7	2.9	1.1
MeGal	1.6	8.9	16.2	19.3	20.1
MeGal2	4.7	9.6	13.6	13.9	13.3
MeGal3	14.8	17.1	16.0	9.9	2.3
MeGal4	79.0	43.9	10.9	3.5	2.9

Example 4: Activity with o-nitrophenyl-ß-D-galactopyranoside (ONPG)

The activity of HIGAL and MTGAL with ONPG was tested by mixing 200 µl (normally 5.5 mg/ml) ONPG in 50mM sodium acetate, 1 mM $CaCl_2$, 0.01% Triton X-100, pH 6.5 with 25 µl galactanase (normally 1 mg/ml) in the well of a microtiter plate. Release of o-nitrophenol (ONP) was measured at room temperature at 405 nm every 10 seconds normally for 30 min on a SpectraMaxPlus (Molecular Devices). Effects on the observed release of ONP was studied with varied enzyme concentration, ONPG concentration and with addition of galactose (Gal), ß-1,4-galactobiose (Gal2) (Megazyme), ß-1,4-galactotriose (Gal3), ß-1,4-galactotetraose (Gal4), glucose (Glu), arabinose (Ara), galacturonic acid (GalA), maltose (Mal) or maltotriose (Mal3).

In Tables 8-11 below, incubation times required to increase the observed absorbance at 405 nm by given amounts are listed. Cells marked 'n.r.' indicate that the increase in absorbance was not reached in the experiment. In general, the initial increase in absorbance at 405 nm was very slow, but after a lag phase the rate of ONP release often increased drastically – often approximately exponentially. The most likely explanation for the observed kinetics is that ONPG reacts with the enzyme to give an enzyme-galactosyl intermediate which hydrolyses very slowly. Instead, the Gal of the intermediate is released by transglycosylation, initially with ONPG or added sugar as acceptor. In cases where the rate of ONP release increases, these transglycosylation products are even better acceptors than the initial ones. As seen in Table 8, the rate of ONP release is about proportional to the amount of added enzyme. HIGAL releases ONP faster than MTGAL at identical enzyme dosage. Addition of Gal (5 mg/ml) is seen to slow the ONP release by about a factor of two for MTGAL and a factor of three for HIGAL. Probably, Gal does not significantly slow formation of the enzyme-galactosyl intermediate, which would accumulate even if Gal had high affinity for the -1 or +1 subsite. More likely, Gal inhibits the subsequent transglycosylation, which requires binding of ONPG to the +1 and +2 subsites, e.g. by binding to the +2 subsite. With 50 mg/ml Gal added (results not shown) release of ONP was even slower with only insignificant increase of absorbance at 405 nm in 30 min.

The results in Table 9 show that rate of ONP release is similar with 5 and 10 mg/ml ONPG but slower at 2.5 and especially 1.25 mg/ml ONPG. This indicates that the rate-limiting transglycosylation reaction with ONPG as acceptor has a Km of about 3 mg/ml.

In Table 10 effects of adding 0.5 or 0.05 mg/ml Gal2, Gal3 or Gal4 are given. Contrary to Gal each of these three galactooligosaccharides increases the rate of ONP release. The initial ONP release rates indicate that Gal4 is more efficient than Gal3 as acceptor, and that Gal3 is more efficient than Gal2. With Gal2 and Gal3, ONP release rate increases significantly with incubation time, indicating that transglycosylation products (initially Gal3 and Gal4, respectively) are more efficient acceptors than the added sugars, whereas the release

rate is relatively constant with Gal4. These results indicate that HIGAL possesses four significant subsites (+1,+2, +3,+4) on the reducing side of the cleaved bond.

In Table 11 results upon addition of Glu, Ara, Mal, Mal3 and GalA are given. As experiments were run on three different days, and ONP release rate even in identical experiments had been seen to vary slightly (possibly due to variants in temperature), results with only ONPG and HIGAL added run in the same three experiments are shown. It is seen that 5 mg/ml Ara inhibits the transglycosylation, resulting in about three times slower ONP release. 5 mg/ml Glu also has slight inhibitory effect, whereas 50 mg/ml Glu (results not shown) resulted in very little ONP release (<0.02) in 30 min. As with Gal, this indicates binding of these sugars to subsites in the enzyme-galactosyl intermediate, which prevents ONPG to act as acceptor and where the sugars themselves also has little or no acceptor fucntion. With 5 mg/ml Mal or Mal3 no significant effects on ONP release are observed. 5 mg/ml GalA has weak inhibitory effect, whereas 50 mg/ml GalA slows ONP release by about a factor two. From these results ranking of the inhibitory effect of the tested sugars is: Gal ~ Ara > Glu > GalA > Mal = Mal3 = 0.

Using HPAE-PAD chromatography (Dionex LC-500 System, PA-100 column, linear gradient of 0-0.6 M sodium acetate in 100 mM NaOH), the production of larger oligosaccharides from transglycosylation upon incubation of HIGAL (110 µg/ml) at room temperature (0.5 to 14 min followed by heat inactivation for 10 min at 95°C resulting in A_{405}: 0.15-0.67) in 50 mM sodium acetate, 1 mM $CaCl_2$, 0.01% Triton X-100, pH 6.5 with ONPG (5 mg/ml) with and without Gal2 (0.05 mg/ml) or Gal3 (0.05 mg/ml) as acceptor was verified.

Table 8: Rate of ONP release, MTGAL and HIGAL in varying dosages, and +/- sugar

Enzyme:		**MTGAL**	**MTGAL**	**MTGAL**	**MTGAL**	**MTGAL**	**HIGAL**	**HIGAL**	**HIGAL**	**HIGAL**	**HIGAL**
µg/ml		**110**	**55**	**28**	**110**	**55**	**110**	**55**	**28**	**110**	**55**
ONPG (5 mg/ml)											
Sugar: mg/ml					Gal: 5	Gal: 5				Gal: 5	Gal: 5
Time (min) to increase A405 by:	0.025	14.0	29.9	40.4	26.9	40.2	9.0	20.4	41.4	32.0	57.0
	0.05	20.5	42.5	n.r.	44.5	n.r.	10.3	22.9	46.5	35.7	n.r.
	0.1	26.4	54.5	n.r.	n.r.	n.r.	11.5	25.4	51.0	41.5	n.r.
	0.2	31.4	n.r.	n.r.	n.r.	n.r.	12.7	28.0	56.5	46.7	n.r.
	0.4	34.5	n.r.	n.r.	n.r.	n.r.	13.8	30.9	n.r.	52.7	n.r.
	0.8	39.2	n.r.	n.r.	n.r.	n.r.	15.0	32.0	n.r.	59.0	n.r.
	1.6	43.9	n.r.	n.r.	n.r.	n.r.	15.0	35.0	n.r.	n.r.	n.r.
	3.2	46.7	n.r.	n.r.	n.r.	n.r.	17.7	37.9	n.r.	n.r.	n.r.

Table 9: Rate of ONP release at varying ONPG concentrations

Enzyme: µg/ml		**HIGAL: 110**	**HIGAL: 110**	**HIGAL: 110**	**HIGAL: 110**
ONPG (mg/ml)		10	5	2.5	1.25
Sugar: mg/ml					
Time (min) to increase A405 by:	0.025	6.3	6.0	9.7	28.7
	0.05	7.7	8.0	11.3	n.r.
	0.1	8.8	9.2	12.7	n.r.
	0.2	10.0	10.2	14.0	n.r.
	0.4	11.2	11.3	15.5	n.r.
	0.8	12.3	12.5	17.3	n.r.
	1.6	13.5	13.8	19.5	n.r.
	3.2	14.7	15.3	22.8	n.r.

Table 10: Rate of ONP release, addition of various amounts of various galactooligosaccharides

Enzyme: µg/ml		**HIGAL: 110**	**HIGAL: 110**	**HIGAL: 110**	**HIGAL: 110**	**HIGAL: 110**	**HIGAL: 110**	**HIGAL: 110**
ONPG (mg/ml)		5	5	5	5	5	5	5
Sugar: mg/ml			Gal2: 0.5	Gal2: 0.05	Gal3: 0.5	Gal3: 0.05	Gal4: 0.5	Gal4: 0.05
Initial rate (mOD/min)		0.9	6	2	200	40	700	80
Time (min) to increase A405 by:	0.025	10.0	2.7	5.5	0.0	0.5	0.0	0.2
	0.05	11.3	3.5	6.8	0.2	1.0	0.0	0.5
	0.1	12.8	4.8	8.2	0.3	1.7	0.0	0.8
	0.2	14.2	6.0	9.5	0.5	2.3	0.2	1.5
	0.4	15.7	7.2	10.7	0.8	3.3	0.5	2.5
	0.8	17.0	8.3	11.8	1.5	4.8	1.0	3.7
	1.6	18.5	9.7	13.3	2.5	6.3	2.2	5.2
	3.2	20.0	11.0	15.2	3.8	7.7	5.3	7.5

Table 11: Rate of ONP release, inhibition by sugars

Enzyme: µg/ml		HIGAL: 110	HIGAL: 110	HIGAL: 110	HIGAL: 110	HIGAL: 110	HIGAL: 110	HIGAL: 110	HIGAL: 110	HIGAL: 110
ONPG (mg/ml)		5	5	5	5	5	5	5	5	5
Sugar: mg/ml			Glu: 5	Ara: 5		Mal: 5	Mal3: 5		GalA: 50	GalA: 5
Time (min) to increase A405 by:	0.025	8.5	12.8	26.5	11.0	11.5	11.0	8.7	13.7	9.5
	0.05	9.5	14.8	n.r.	12.5	13.0	12.3	9.8	16.7	11.2
	0.1	10.8	17.0	n.r.	13.8	14.3	13.8	11.0	20.0	12.7
	0.2	12.0	19.3	n.r.	15.2	15.7	15.2	12.2	23.3	14.3
	0.4	13.3	21.8	n.r.	16.5	17.2	16.5	13.3	26.8	15.8
	0.8	14.5	24.3	n.r.	18.0	18.7	18.0	14.7	n.r.	17.5
	1.6	15.8	27.0	n.r.	19.5	20.2	19.3	16.0	n.r.	19.3
	3.2	17.3	n.r.	n.r.	21.3	22.0	21.3	17.3	n.r.	22.5

Example 5: Activity on lactose

HIGAL (60 µg/ml) and MTGAL (750 µg/ml) were incubated at 50°C with lactose (Lac) (100 mg/ml) at pH 4.8 (25 mM sodium citrate), 6.45 (25 mM sodium acetate, 0.5 mM $CaCl_2$, 0.005% Triton X-100) and 8.6 (50 mM Tris, 0.01 % Brij 35). 20 µl samples were withdrawn after 2, 23 and 120 hours, 980 water added and enzyme inactivated by heating to 95°C for 10 min. After a further 20 time dilution with water, samples were analysed using HPAE-PAD (Dionex LC-500 system, PA-100 column, 0-3 min: 150 mM NaOH, 3-19 min: linear gradient 0-0.18 M sodium acetate in 150 mM NaOH). Response factors for the individual peaks were estimated from standards of Gal, Glu, Lac, Gal2, Gal3 and Gal4.

Under these conditions only MTGAL at pH 4.5 and 6.5 gave significant conversion of Lac. In Tables 12 and 13 weight fractions of the analysed products with MTGAL at pH 4.5 and 6.45 are given. The figures indicate weight% of the products resulting from the incubation. The term DP3 indicates transglycosylation product consisting of three sugar units, and the term DP4+ transglycosylation products consisting of four or more sugar units. Unfortunately, the analysis method used was not able to separate Glu and Gal.

With transglycosylation occurring according to the reaction:

2 Lac -> DP3 (=Gal_2Glu) + Glu

the weight fraction of DP3 should be about three times higher than the weight of the monomer. After 2 hours the ratio is about 1.5 at both pH's indicating that this is not the only reaction taking place. The production of larger oligosaccharides (DP4+) is a result of the initial transglycosylation product functioning as acceptor for further transglycosylation:

Lac + DP3 -> DP4 + Glu

Also, from the increasing amount of Gal/Glu without corresponding increase in transglycosylation products (DP3 and DP4+) after 23 and 120 hours, it is evident that hydrolysis of transglycosylation products takes place. These hydrolysis reactions seem to be slower at pH 6.45 than at pH 4.5.

Table 12: Activity of MTGAL on lactose (pH 4.5)

Incubation time (hours)	**2**	**23**	**120**
Glu/Gal	1.4	12.5	38.8
Lac/Gal2	96.0	78.7	51.2
DP3	2.0	7.0	8.9
DP4+	0.5	1.8	1.1

Table 13: Activity of MTGAL on lactose (pH 6.45)

Incubation time (hours)	2	23	120
Glu/Gal	1.0	6.5	21.0
Lac/Gal2	95.7	85.3	62.6
DP3	1.4	6.3	11.7
DP4+	1.9	1.9	4.7

Example 6: Activity on galactan

Lupin galactan (Megazyme) was incubated with BLGAL (0.1-10 µg/ml) at pH 6.5 (50 mM MES, 1mM CaCl2) and with AAGAL (0.1-10 µg/ml) at pH 4.0 (50 mM sodium acetate, 1 mM CaCl2) at 30°C. Samples were withdrawn after 45 min to 24 hours and enzyme inactivated by heating to 95°C for 10 min. Reaction products were analysed using HPAEC-PAD on a Dionex chromatographic system using a CarboPac PA-100 column and a linear gradient 0 to 0.3 M sodium acetate in 0.15 M NaOH. Purified galacto-oligosaccharides were used to identify products.

With BLGAL the initial main product is galactotetraose with both smaller and larger oligomers also present. Upon longer incubation the fractions of galactose, galactobiose and galactotriose increase and after prolonged incubation only these three oligomers are seen in molar ratios of about 1:0.4:0.9.

AAGAL initially produces a more homogeneous mixture of galactooligomers. Further degradation yields mainly galactose, galactobiose and galactotriose, and finally almost exclusively galactose and galactobiose are seen in a molar ratio of about 2:1. Small peaks probably corresponding to galactobioses and galactotrioses resulting from transglycosylation reactions with glucosidic bonds different from ß-1,4 are also present.